Diagnostic Mutation Profiling and Validation of Non–Small-Cell Lung Cancer Small Biopsy Samples using a High Throughput Platform

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Background: A single platform designed for the synchronous screening of multiple mutations can potentially enable molecular profiling in samples of limited tumor tissue. This approach is ideal for the assessment of advanced non–small-cell lung cancer (NSCLC) diagnostic specimens, which often comprise small biopsies. Therefore, we aimed in this study to validate the mass spectrometry-based Sequenom LungCarta panel and MassARRAY platform using DNA extracted from a single 5 μM formalin-fixed paraffin-embedded tissue section.

Methods: Mutations, including those with an equivocal spectrum, detected in 90 cases of NSCLC (72 lung biopsies, 13 metastatic tissue biopsies, three resections, and two cytology samples) were validated by a combination of standard sequencing techniques, immunohistochemical staining for p53 protein, and next-generation sequencing with the TruSight Tumor panel.

Results: Fifty-five mutations were diagnosed in 47 cases (52%) in the following genes: *TP53* (22), *KRAS* (15), *EGFR* (5), *MET* (3), *PIK3CA* (3), *STK11* (2), *NRF-2* (2), *EPHA5* (1), *EPHA3* (1), and *MAP2K1* (1). Of the 90 samples, one failed testing due to poor quality DNA. An additional 7 *TP53* mutations were detected by next-generation sequencing, which facilitated the interpretation of p53 immunohistochemistry but required 5 × 10 μM tumor sections per sample tested.

Conclusions: The LungCarta panel is a sensitive method of screening for multiple alterations (214 mutations across 26 genes) and which optimizes the use of limited amounts of tumor DNA isolated from small specimens.

Key Words: Non–small-cell lung cancer, Mutation, Mass spectrometry, Biopsy.

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Non–small-cell lung cancer (NSCLC), a disease which accounts for over 85% of all lung cancer cases,[1] is currently evolving into a collection of molecular subtypes with individual "personalized" therapies[2] and clinicopathological associations. Increases in progression-free survival of more than 4 months, compared with standard chemotherapy regimens, have been observed after treatment with epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKIs) in patients harboring the appropriate activating mutation or translocation.[3–5] Consequently, screening for these alterations is becoming an established component of routine diagnostic practice worldwide. There are, however, a multitude of other targets[6] emerging in both adenocarcinoma (ADC) and squamous cell carcinoma (SCC), the two main subtypes of NSCLC, and the possibility of exploiting alterations, including those in the MAP2K[7,8] and mTOR[9,10] pathways has led to the development of comprehensive assays designed to detect multiple mutations in different genes.

The majority of NSCLC cases present at a late stage with an associated poor prognosis.[11] As a result up to 70% of lung tumors are sampled and staged either by cytological specimens (such as fluids or lymph node aspirates) or by small tissue biopsies and not by resection samples.[12] These specimens will often have a lower percentage of cancer cells and due to the small biopsy volume a limited number of sections available for genetic testing. Therefore, the introduction of

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high throughput platforms with the capacity to screen multiple mutations from DNA derived from a single section will have a significant impact on diagnostic screening. Although next-generation sequencing (NGS) can potentially screen for an expanded array of mutations, copy number alterations and translocations, the diagnostic yield is often restricted by the need for a relatively high neoplastic cell content (NCC) required to give an optimal DNA quality and quantity. Currently, this can often only be obtained from a resection specimen. In this study, we aimed to evaluate the mass spectrometry-based LungCarta panel on a series of NSCLC samples which comprised mainly small biopsies from patients with (mostly) advanced stage disease.

PATIENTS AND METHODS

Patients and Tissue Specimens

Ninety samples comprising formalin-fixed paraffin-embedded tissue (FFPET) biopsies and cytology specimens, with accompanying reports, were reviewed from patients diagnosed with NSCLC in Greater Manchester, UK over a period extending from 2008 to 2012. Samples were fixed in 10% neutral-buffered formalin for a period of 12 to 24 hours. Tumor morphology (based on a haematoxylin and eosin stain) was reviewed by two pathologists, (A.M.Q. and D.N.). ADCs and SCCs were diagnosed according to established respective criteria of glandular differentiation or keratinization.[12–14] Tumors with definite ADC or SCC features were classified on morphology alone, (58 cases) and the remaining 32 cases were categorized based on thyroid transcription factor-1 (TTF-1) and p40 immunohistochemical staining. Tumors that were TTF-1 positive and p40 negative were classified as ADC, whereas those that were p40-positive and TTF-1 negative were diagnosed as SCC. Any tumors that were negative for both stains were classified as NSCLC not otherwise specified (NOS; there were no cases with positivity for both stains). The NCC of each sample was determined based on the number of tumor nuclei relative to nuclei of inflammatory cells and stromal cells, and categorized as less than 10%, 10–20%, 20–30%, 30–50%, or more than 50%. Any samples with less than 20% NCC were macrodissected to increase the final NCC to at least 10%. The tissue surface areas ranged from 2 to 300 mm^2 (median 12 mm^2). This study was approved by the North West Research Ethics Committee (National Research Ethics Service), reference numbers 07/H1014/96 and 09/H1011/55. All molecular and immunohistochemical tests were conducted in accredited clinical genetics and pathology laboratories.

Mutation Profiling and Confirmation

Screening for mutations was performed with the LungCarta Panel (Sequenom, San Diego, CA), which enables the detection of 214 mutations across 26 genes (*AKT1, ALK, BRAF, DDR2, EGFR, EPHA3, EPHA5, ERBB2, FGFR4, JAK2, KRAS, MAP2K1, MET, NOTCH1, NRAS, NRF2, NTRK1, NTRK2, NTRK3, PIK3CA, PTCH1, PTEN, PTPN11, PTPRD, STK11, TP53*). In each case, DNA was extracted manually from one 5 µM FFPET section or curl using the cobas DNA Sample Preparation Kit. For cytology samples, the DNA was extracted from one 5 µM section of paraffin-embedded cell block preparations. DNA concentrations were assessed by optical density measurement on a NanoDrop spectrophotometer, and ranged from 0.55 to 112 ng/µl (median 7.31 ng/µl). Mutation profiling was conducted using the Sequenom MassARRAY platform, which utilizes multiplexed polymerase chain reaction (PCR) single base extension reactions (iPLEX chemistry)[15] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.[16–18] The LungCarta assay had previously been validated on the Sequenom platform using known mutation positive control samples, previously characterized on other platforms, e.g., *KRAS* mutation on Pyrosequencing. This allowed us to set the threshold levels for confident assignment of mutations. Equivocal mutations were defined as spectral alterations detected which were of uncertain significance. Mutations were detected using DNA quantities, which ranged from 28.6 to 5850 ng (median 380.1 ng).

Mutations identified using LungCarta were confirmed by various methods including Sanger sequencing, pyrosequencing, the cobas EGFR Mutation Test, NGS (TruSight Tumor panel, Illumina, San Diego, CA),[19,20] or retesting by the LungCarta assay (Fig 1). NGS with the TruSight Tumor panel was also used to screen for the presence of *TP53* mutations in samples which were wild-type for *TP53* alterations detected

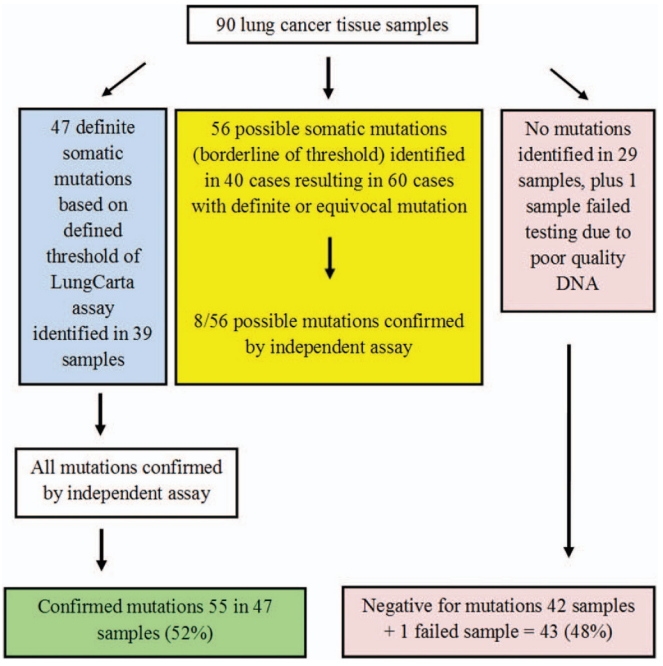

FIGURE 1. Flowchart of mutations detected by LungCarta panel including the confirmations of all those with definite mutant allele spectra and those which appeared equivocal due to low allele frequency (less than 10%) or a poor quality spectrum. Samples with *TP53* mutations were also confirmed by p53 immunohistochemistry. One sample failed the LungCarta assay due to poor quality DNA and low cancer cell content. Six mutations (see text), accounting for 11% of 55 mutations finally reported, were present on the initial assay and were not confirmed on repeat screening by LungCarta due to a low amplification yield or equivocal spectra.

by the LungCarta panel and which had p53 protein expression detected by immunohistochemistry (IHC). The TruSight panel screens 82 exons in 26 genes across 175 amplicons (*AKT1, ALK, APC, BRAF, CDH1, CTNNB1, EGFR, ERBB2, FBXW7, FGFR2, FOXL2, GNAQ, GNAS, KIT, KRAS, MAP2K1, MET, MSH6, NRAS, PDGFRA, PIK3CA, PTEN, SMAD4, SRC, STK11, TP53*), including complete coverage of greater than 85% of exon coding sequence and total coverage of the coding sequence of selected genes such as *TP53*. For the TruSight panel DNA was extracted from 5 × 10 μM sections of selected cases using the Qiagen FFPET kit followed by a qPCR-based assay to quality assess the extracted material for further processing. An extension and ligation-based amplicon library preparation assay specific for each of the two strands of DNA was used for all targets and the index sequence was incorporated into the tailed universal PCR primers to identify the sample. The resulting two independent libraries were combined for sequencing. NGS was carried out using Illumina MiSeq[21] (San Diego, CA) with a minimum read depth of at least 1000× combined coverage between the two libraries targeted for all amplicons. The information from both strands was used to demultiplex and align the reads and calculate variant frequencies.[19]

Immunohistochemistry

All IHC was conducted on 5 μM FFPET sections. Appropriate positive and negative controls were included with the study sections. Diagnostic IHC stains (TTF-1 and p40) were carried out on 3 μM sections from a total of 32 cases, where the morphology based on review of the H&E stain was inconclusive. Antibody incubations and detection were carried out on a Menarini IntelliPATH FLX (A. Menarini Diagnostics, Winnersh-Wokingham, Berkshire, UK) using TTF-1 (mouse monoclonal 8G7G3/1, 1:200, Dako, Glostrup, Denmark) or ΔNp63 (rabbit polyclonal p40, 1:200, Calbiochem, Nottingham, UK) as described previously.[22] TTF-1 and p40 staining was nuclear.

Immunohistochemical staining for ALK protein was carried out on a section from each ADC and NOS case (46 cases in total). Antigen retrieval was performed by heating slides for 30 minutes at 100°C in Bond Epitope Retrieval Solution 2 (Leica Microsystems, Milton Keynes, UK), followed by a peroxidase blocking step for 5 minutes. The sections were then incubated with anti-ALK antibody (clone 5A4, Novocastra 1:25). Sections were stained with the Leica BOND-MAX autostainer (Leica Microsystems), Bond Polymer Refine Detection kit (Leica) and Leica Microsystems reagents, and were assessed for cytoplasmic staining.

Staining for p53 protein was carried out using a mouse monoclonal antibody (clone DO-7, Dako, 1:50) on 19 of 20 cases with a *TP53* mutation detected by LungCarta screening (one case with a *TP53* mutation did not have available tissue). The p53 staining was nuclear, and was also conducted on 10 cases wild-type for *TP53* by LungCarta screening. Sections were stained with the automated Ventana BenchMark XT IHC /ISH Staining Module (Ventana Co., Tucson, AZ) as described previously.[23] *H* scores ranging from 0 to 300 were calculated as intensity × %tumor area stained (area of tumor stained × intensity score 1+) + (area of tumor stained × intensity score 2+) + (area of tumor stained × intensity score 3+).[24]

Statistical Analysis

Kaplan–Meier survival curves were conducted with MedCalc version 11.4.40 (Mariakerke, Belgium). The curves were compared using the log rank test. The χ^2 test was used to analyze the relation between categorical variables and the Student *t* test was used for continuous variables. All *p* values were two-sided and a *p* value less than 0.05 was considered significant.

RESULTS

Patient Characteristics

A summary of clinical and pathological characteristics is provided in Table 1. A total of 90 cases which included 72 lung biopsies, three resections, 13 metastatic tissue samples, and two cytology specimens were reviewed and classified as 40 ADC, 44 SCCs, and six NSCLC NOS. All ADC and NOS cases with adequate tissue were screened for ALK protein expression by IHC. A total of 44 cases were stained (two cases had insufficient tissue available) all of which were negative for ALK expression. The majority of the patients was either current or former smokers (67, 74%) and had advanced NSCLC (TNM Stage III or IV) with a median overall survival time of 312.5 days (range 34–1961 days). Individuals who were former smokers ($n = 48$) had a better survival than current smokers ($n = 24$; $p = 0.006$; Supplementary Figure 1A, Supplemental Digital Content 1, http://links.lww.com/JTO/A789). Overall survival outcomes were improved in those with early stage disease (Stage I or II) compared with those with Stage III or Stage IV disease ($p = 0.04$; Supplementary Figure 1B, Supplemental Digital Content 2, http://links.lww.com/JTO/A790).

Survival outcomes did not differ between patients diagnosed with ADC or SCC ($p = 0.6$). There was no difference in overall survival between men and women ($p = 0.4$), or between those with well-moderately differentiated and poorly differentiated SCCs ($p = 0.6$).

Mutation Detection by LungCarta and Verification

Ninety samples were tested of which there was complete failure in only one due to poor quality DNA. The median success rate for an individual genotype in the panel was 95.5%. On initial screening with the LungCarta panel 39 of 90 samples (43%), 47 definite mutations were identified. Equivocal mutations were defined as those with low mutant allele frequency (less than 10%), or poor quality spectra. An additional 56 equivocal mutations were detected in 40 cases (17 cases with definite mutation and 23 cases with equivocal only), resulting in 60/90 samples (67%) with either definite or equivocal mutations.

To verify mutations (definite and equivocal), a combination of pyrosequencing, Sanger sequencing, sequencing with the cobas EGFR Mutation Test, next-generation sequencing (TruSight Tumor Panel), and rescreening by the LungCarta panel was used. Using this detection limit, 31 of 56 equivocal mutations with low allele frequency alterations (less than 10%) were excluded from further testing (reported as negative

TABLE 1. Clinicopathological Features of Patients and Samples, $n = 90$

Median age, years (range)	65 (41–85)
Sex, no. (%)	
Men	54 (60)
Women	36 (40)
Smoking status, no. (%)	
Current smoker	24 (27)
Former smoker	48 (53)
Never smoker	2 (2)
Unknown	16 (18)
Histology, no. (%)	
Adenocarcinoma	40 (44)
Squamous cell carcinoma	44 (49)
NOS	6 (7)
Type of specimen, no. (%)	
Primary; lung biopsies	72 (80)
Wedge resection	1 (1)
Lobectomies	2 (2)
Metastatic; lymph nodes	7 (8)
Pleural biopsies	4 (5)
Pleural fluid	1 (1)
Pericardial effusion	1 (1)
Brain	1 (1)
Soft tissue	1 (1)
Stage[a], no. (%)	
I or II	6 (7)
IIIA	18 (20)
IIIB	18 (20)
IV	31 (34)
Unknown	17 (19)

[a]Staging by TNM 7th edition.[49]
NOS, not otherwise specified.

for mutations). Of the remaining 25 equivocal mutations further testing confirmed eight mutations (Fig. 1). This yielded a final total of 55 confirmed mutations in 47 samples.

There were six samples with a mutation meeting the threshold to be defined as a definite mutation on primary screening for which the repeat (confirmation) MassARRAY spectrum was inconclusive. These included three mutations which had a low amplification yield from the amplification stage of the assay (*EPHA5* p.E503K, *STK11* p.F354L, and *TP53* p.R248W). In addition, an equivocal spectrum was reported for the repeat *EPHA3* p.D806N test and one repeat assay for an *STK11* F354L mutation failed. Of note is that one *TP53* mutation (p.R175H) identified on the original assay with two comutations (*TP53* p.G245S, *STK11* p.Y272Y) was not present on repeat assay although the comutations were detected.

To further verify the presence of *TP53* mutations, we carried out immunohistochemical staining for p53 protein on 19 *TP53*-mutated samples (one of 20 samples harboring a *TP53* mutation did not have sufficient tissue for IHC). It was important to determine the accuracy of the LungCarta genotyping for *TP53* because more mutations were detected in this gene than any of the others genotyped. As *TP53* is a large gene and multiple somatic mutations have been described, we decided to use IHC to determine protein levels as this is available as a standard diagnostic assay. Tumor staining was graded by a modified *H* score[24,25] (maximum possible score 300, Supplementary Figure 2, Supplemental Digital Content 3, http://links.lww.com/JTO/A791) and the staining intensities were compared with those of 10 samples which were wild-type for *TP53*.

TP53-mutated samples had either strong (3+) or moderate (2+) staining intensities with *H* scores, which ranged from 70 to 240 (mean 172 ± 46 SD, median 170). Only three of 19 tumors had scores of 120 or less (70, 100, and 120). Although tumors reported negative for *TP53* mutations after LungCarta screening tended to have lower staining intensities, the range was variable extending from 10 to 250 (mean 120 ± 87 SD, median 120). In fact, there were a number of these samples, which had significant staining (*H* score values of 90, 120, 120 170, 210, and 250, respectively). We hypothesized that there were undetected mutations present which were responsible for these high intensity protein levels. As Sanger sequencing would have required more DNA than was available and is more labor intensive, the 10 cases wild-type for *TP53* by LungCarta were screened by the TruSight tumor panel, which provides complete coverage of the *TP53* sequence. The IHC *H* scores and the mutations detected by both panels from these 10 samples are listed in Table 2. There were four cases with relatively high *H* scores (90, 120, 170, and 210) which had a missense mutation of *TP53* on NGS. In addition, three tumors with low *H* scores of 12, 5, and 0 were found to harbor a *TP53* null mutation, frameshift mutation, and splice site mutation, respectively. All seven of these mutations were not included in the LungCarta panel. There were two samples with strong IHC staining (*H* scores 250 and 120) and one tumor with focal weak staining (*H* score 10) with no *TP53* mutation. The presence of a missense mutation was strongly associated with any degree of moderate to strong IHC staining (*H* score greater than 20; $p < 0.0001$). NGS of these 10 selected cases did not detect any additional mutations that were already screened for in the LungCarta panel, indicating that there were no false negatives with the LungCarta assay.

Associations of Mutations identified by LungCarta with Clinicopathological Characteristics and NCC

A total 55 unequivocal mutations were detected in 47 of 90 NSCLC tumor samples (52% of samples with at least one confirmed mutation). These included 22 alterations of *TP53* (24% of samples), 15 *KRAS* (17%), five *EGFR* (6%), three *MET* (3%), three *PIK3CA* (3%), two *STK11* (2%), two *NRF-2* (2%), one *EPHA5* (1%), one *EPHA3* (1%), and one *MAP2K1* (1%). The individual mutations reported are listed in Table 3. A comparison of mutations reported in histological subgroups (Fig. 2) revealed that 22 of 44 SCCs (50%) harbored alterations comprising *TP53* (12, 27%), *KRAS* (three, 7%), *PIK3CA* (three, 7%), *NRF-2* (two, 5%), *STK11* (two, 5%), *EPHA5* (one, 2%), and *EGFR* (1, 2%), whereas 21 of 40 ADCs (52%)

TABLE 2. Comparison of p53 Immunohistochemistry *H* Scores with Mutations Detected by the TruSight Tumor Panel in 10 Samples Negative for *TP53* Mutation by the LungCarta Panel

Mutations Detected by LungCarta (Variant Frequency %)	Mutations Detected by TruSight (Variant Frequency %)	TP53 IHC *H* Score[a]
KRAS G12A (75.0)	KRAS G12A (73.1), APC Q1127[b] (4.3)	250
KRAS G12V (21.8)	TP53 Q144P (23.8), KRAS G12V (29.4), FBXW7 R505L (14.1)	210
None	TP53 G245C (12.2)	170
MET N375S (83.8) KRAS G12V (73.9)	TP53 G334V (70.8), KRAS G12V (77.1)	120
None	KRAS G12C (3.3)	120
None	TP53 H179R (23.2)	90
None	TP53 E258[b] (69.8)	12
None	None	10
None	TP53 E298fs (41.5), PTEN V45fs (35.0), SRC V474M (4.0)	5
None	TP53 c.673-1 splice (87.1)	0

[a]*H* score calculated from (area of tumor stained × intensity score 1+) + (area of tumor stained × intensity score 2+) + (area of tumor stained × intensity score 3+); maximum score 300.
[b]Null mutation.
IHC, immunohistochemistry.

were diagnosed with mutations (Table 3) including *TP53* (six, 15%), *KRAS* (ten, 25%), *EGFR* (three, 7.5%), *MET* (two, 5%), *MAP2K1* (one, 3%), and *EPHA3* (one, 3%). There were six mutations (three *TP53*, two *KRAS*, and one *MET* mutation/variant) reported in four of six cases finally classified as NSCLC NOS.

The most common alteration reported was in *TP53* with 22 mutations detected in 20 tumor samples, followed by *KRAS* (15 mutations in 15 samples) and *EGFR* (five mutations in four samples). *KRAS* mutations were associated with ADC histology ($p = 0.03$, Table 4). There was no apparent correlation between age, sex, smoking history, SCC differentiation, or type of specimen (primary or metastatic) and the overall mutation status, the presence of *TP53* or *KRAS* mutations (Table 4). Multiple mutations were reported in samples from six patients (6.7%), three of which included a *KRAS* mutation (Supplementary Table 1, Supplemental Digital Content 4, http://links.lww.com/JTO/A792). The presence of any detected mutations ($p = 0.75$), *TP53* mutations ($p = 0.22$), or *KRAS* mutations ($p = 0.64$) detected by the LungCarta panel was not significantly associated with survival.

Thirty-four samples (38%) presented with necrosis accounting for up to 50% of the tissue content; however, this did not appear to interfere with subsequent amplification reactions. The distribution of samples according to estimated NCC groups included 13 cases (14%) with 10–20%, 27 cases (30%) with 20–30%, 33 cases (37%) with 30–50%, and 17 cases (19%) with over 50% NCC. A total of 38 cases (including those originally with NCC below 10%) were macrodissected to increase the final NCC. The minimum NCC of samples in which mutations were detected was 10%. There was no significant difference between the number of alterations reported in the different NCC groups (eight mutations in the 10–20% group, 13 mutations in the 20–30% group, 20 mutations in the 30–50% group, and six mutations diagnosed in the over 50% group; $p = 0.6$) using χ^2 analysis (Supplementary Figure 3, Supplemental Digital Content 5, http://links.lww.com/JTO/A793). The minimum number of neoplastic cells present, in samples where a mutation was detected, was estimated as 250.

DISCUSSION

We have screened 90 NSCLC tumor samples, the majority of which were small biopsy specimens from patients with advanced stage disease, using the Sequenom LungCarta platform. A multiplexed method such as the MassARRAY system allows the screening of multiple mutations in a single diagnostic assay from one FFPET 5 μM section. Genetic alterations were confirmed in 52% of samples, (52% of ADCs and 50% of SCCs). Of the 55 mutations detected, 49 of these (89%) were confirmed by either an alternative method or a repeat screening by the LungCarta panel.

The *TP53* tumor suppressor gene harbored the greatest number of mutations in both ADCs and SCCs, consistent with reported rates of *TP53* mutations in 36 to 50% of ADCs[26,27] and in up to 80% of SCCs.[28,29] Using p53 protein IHC, we determined that the majority of samples (16 of 19) with a *TP53* mutation detected by LungCarta were strongly positive for p53 expression (*H* scores greater than 120), whereas the other three had moderate staining intensities. The LungCarta panel screens for 28 *TP53* missense mutations that are often detected at a high frequency.[30] However, our study revealed that six of 10 samples *TP53* wild-type by LungCarta screening had significant staining intensities, and four of these were found by NGS to harbor missense mutations not represented on the LungCarta panel. There were four tumors wild-type for *TP53* (by LungCarta screening) which had low *H* scores of less than or equal to 12. These included two carcinomas with *TP53* nonsense and frameshift mutations detected by NGS, which would be expected to have an absent or reduced intensity of staining due to a lack of discernible protein. The IHC staining intensity significantly correlated with detection of a *TP53* missense mutation and may be a more practical screening tool for identification of p53 mutant NSCLC due to the large numbers of potential p53 mutations.

TABLE 3. Mutations detected by LungCarta panel in 90 NSCLC Tumor Samples

Gene	No. (%) of Samples Mutated				Total No. (%) of All Mutations Detected	Mutations Detected
	Total	ADC	SCC	NOS		
TP53	20 (22.2)	6 (15)	12 (27)	2 (33)	22 (40)	4 x V157F, 2 x R158L, 2 x Y220C, 2 x R248W, 2 x R273C, R158C, R158P, R175H, R175L, G245S, G245V, R248L, R249M, R273H, R282W
KRAS	15 (17)	10 (25)	3 (7)	1 (17)	15 (27.2)	6 x Gly12C, 6 x G12V, G12A, G13D, Q61H
EGFR	4 (4.4)	3 (7.5)	1 (2)	0 (0)	5 (9)	G746_S752delinsV, V774M, G746_A750del, L858R
MET	3 (3.3)	2 (5)	0 (0)	1 (17)	3 (5.5)	3 x N375S[a]
PIK3CA	3 (3.3)	0 (0)	3 (7)	0 (0)	3 (5.5)	2 x E545K, H1047R
STK11	2 (2.2)	0 (0)	2 (5)	0 (0)	2 (3.6)	Y272Y[b], F354L
NRF-2	2 (2.2)	0 (0)	2 (5)	0 (0)	2 (3.6)	R34Q, D29H
MAP2K1	1 (1.1)	1 (3)	0 (0)	0 (0)	1 (1.8)	Q56P
EPHA5	1 (1.1)	0 (0)	1 (2)	0 (0)	1 (1.8)	E503K
EPHA3	1 (1.1)	1 (3)	0 (0)	0 (0)	1 (1.8)	D806N

[a]The N375S variant has been reported as a germline missense substitution.[50]
[b]The Y272Y variant has been reported as a silent substitution c.816C>T.[51]
NSCLC, non–small-cell lung cancer; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NOS, not otherwise specified.

Mutations in *KRAS* were the second largest group of mutations identified in this analysis belonged to the *KRAS* gene. *KRAS* mutations were significantly associated with ADC histology and present in 25% of this subgroup, a finding in keeping with elevated rates of *KRAS* mutations in lung ADCs from smoking populations with Caucasian ethnicity.[31,32]

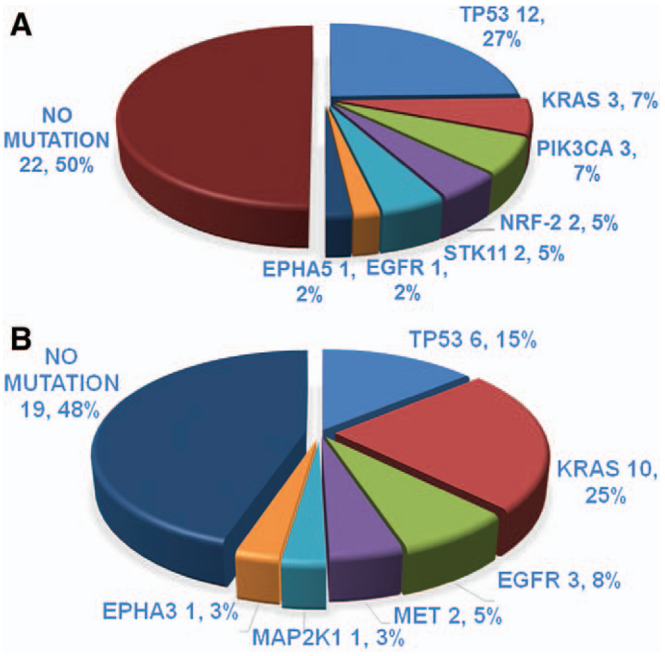

FIGURE 2. Mutations detected by LungCarta panel in (*A*) squamous cell carcinomas, $n = 44$ and (*B*) adenocarcinomas, $n = 40$ (six additional mutations were confirmed in four of six cases classified as non–small-cell lung cancer not otherwise specified, not shown).

The *EGFR* mutant (E746_S752delinsV), finally classified as a squamous tumor, was poorly differentiated on H&E morphology, had diffusely positive p40 staining and was negative for TTF-1 expression by IHC. *EGFR* mutations of SCCs are rare and occur at a rate of less than 5% within this morphological subgroup.[33] Although *EGFR*-mutated SCCs are associated with a lower response rate (25% compared with 70% for ADC) to TKIs[34,35] a multiplexed screening method applicable to all NSCLC could potentially identify these rare alterations enabling the potential selection of patients with SCC for EGFR TKI therapy. One ADC was also found to harbor a mutation of the *EPHA3* gene D806N which has previously been identified in a colorectal ADC.[36] This is the first report of this mutation in lung ADC. Two poorly differentiated SCCs harbored mutations in the nuclear factor (erythroid-derived 2)-like 2 gene (*NRF2*). In keeping with our findings, mutations have been previously associated with squamous histology from men with NSCLC.[37]

We have confirmed the presence of mutations in 47 NSCLC tumor samples tested (52%), most of which are small biopsy samples. There was no apparent difference between the numbers of mutations detected in samples of differing NCC, including the group with the lowest tumor content, which was estimated as 10–20%. This reflects an estimated allele frequency detection limit of 5% for the LungCarta assay, and is comparable with the estimated allele frequency level that can be confidently detected by the SNaPshot system (approximately 5%).[38] The median DNA content of samples assayed was 380.1 ng. Established diagnostic assays such as Pyrosequencing, Sanger sequencing, and the Cobas assay are restricted to the detection of a limited number of mutations per method. The DNA requirement for each of these tests (e.g., the Cobas EGFR Mutation Test is estimated to require a minimum of 150 ng of DNA) implies that an average lung

TABLE 4. Comparison of Mutated Samples with Clinicopathological Factors

	Total No. Mutated Samples (%)			*TP53*-Mutated Samples (%)			*KRAS*-Mutated Samples (%)		
	Pos.	Neg.	*p*	Pos.	Neg.	*p*	Pos.	Neg.	*p*
Median age at diagnosis	65.15	66.48	0.678	68.00	65.27	0.946	63.54	65.99	0.536
Sex									
Men	29 (53)	26 (47)	0.904	12 (22)	43 (78)	0.908	6 (11)	49 (89)	0.066
Women	18 (51)	17 (49)		8 (23)	27 (77)		9 (26)	26 (74)	
Smoking history									
Current/former	37 (51)	35 (49)	0.752	18 (25)	54 (75)	0.205	10 (14)	62 (86)	0.157
Unknown/never	10 (56)	8 (44)		2 (11)	16 (89)		5 (28)	13 (72)	
Histology[a]									
ADC	21 (53)	19 (47)	0.819	6 (15)	34 (85)	0.171	10 (25)	30 (75)	0.0214
SCC	22 (50)	22 (50)		12 (27)	32 (73)		3 (7)	41 (93)	
SCC differentiation									
Low-grade SCC	9 (41)	13 (59)	0.228	7 (32)	15 (68)	0.498	N/A	N/A	
High-grade SCC	13 (59)	9 (41)		5 (23)	17 (77)				
Type of specimen									
Primary	40 (53)	35 (47)	0.637	17 (23)	58 (77)	0.821	11 (51)	64 (51)	0.255
Metastatic	7 (47)	8 (53)		3 (20)	12 (80)		4 (51)	11 (51)	

[a]Data on six NSCLC NOS samples were not included.
ADC, adenocarcinoma; SCC, squamous cell carcinoma; N/A, not applicable; NSCLC, non–small-cell lung cancer; NOS, not otherwise specified.

biopsy will have insufficient material using a combination of methods to provide a profile of mutations.

The advent of targeted NGS panels promises the capacity to diagnose variants with allele frequencies as low as 1%.[39,40] However, caution must be applied in the separation of amplification-derived artifacts from true low frequency variants, and the appropriate limits of detection must be robustly defined for use in a clinical diagnostic setting.[41] The majority of published NGS studies profiling tumor samples have sampled fresh-frozen tissue from primary resection specimens with relatively high NCC levels of at least 50%.[26,29] In this study, we observed that to obtain an adequate DNA yield for NGS it was necessary to use increased number of tumor sections of greater thickness (5 × 10 μM) compared with that required for the Sequenom assay (1 × 5 μM). This seems to be in agreement with data from Tuononen et al. who reported a high concordance rate (96.3–100%) between mutations detected by real time PCR and NGS. This group extracted DNA from 2/3 × 16 μM sections with a NCC of at least 20%, and used 2–3 μg of DNA for sequencing with the Illumina HISeq2000 sequencer.[42] Similarly, a recent report by de Biase et al. describes the application of 454 NGS to cytology samples using 6 × 10 μM macrodissected sections. Another strategy employed to increase the likelihood of detecting low frequency variants in cytology samples and biopsies with NCCs of 5–10% has been to use a twostep DNA amplification protocol.[40] This group also used 454 NGS and noted a positive correlation between NCC and allele frequency. Ultimately the requirement of a greater number of thicker sections containing viable tumor is a challenge facing diagnostic pathology laboratories that may not always be possible to fulfill, but can potentially be met by the implementation of strict algorithms that balance the demands of diagnostic molecular testing.[13]

The majority of previous studies employing systematic testing or multiplexed methods have relied on tumor resection samples from ADCs (Supplementary Table 2, Supplemental Digital Content 6, http://links.lww.com/JTO/A794). Our rate of detection in 52% of cases is comparable with those reported in diagnostic mutation studies, and confirms that small biopsy samples are a valid option for high throughput assays.[43,44] Maeng et al.[44] screened for mutations in biopsy samples obtained with radiological intervention, bronchoscopy or endobronchial ultrasound guidance and reported alterations in 67% of cases. This relatively high mutation rate may be explained by the fact that DNA was extracted from fresh-frozen tissues, avoiding the risk of artifacts that may arise from formalin-fixation. Furthermore, screening of specific oncogenes in selected populations, such as ADCs from Asian never smokers, will result in a higher mutation detection rate (Supplementary Table 2, Supplemental Digital Content 6, http://links.lww.com/JTO/A794).[45–47] We have also confirmed the presence of multiple alterations in 6.7% of tumors screened, a value in agreement with earlier reports such as the percentages of cases with synchronous mutations reported by Yip et al.[17] (8.8% screening with the Sequenom OncoCarta panel) and Okamoto et al.[43] (10.2% screening with the Sequenom LungCarta panel; Supplementary Table 2, Supplemental Digital Content 6, http://links.lww.com/JTO/A794). This highlights the importance of validating high throughput platforms that not only facilitate the optimization of individual treatment regimens but also enable the prediction of resistance to therapies that arise secondary to coexisting alterations.[48]

In our laboratory, the diagnostic turnaround time for the LungCarta assay is 10 working days, with a cost equivalent to approximately two single-plex somatic mutation assays. This

is, therefore, cheaper than undertaking analysis on three or more genes individually. In summary, we report the Sequenom LungCarta panel as a clinically useful diagnostic screening test for small biopsy samples of NSCLC. This assay can confidently detect mutations at NCC levels of 10% and above, using a single 5 μM section.

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